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Epstein-Barr Virus LF2: an Antagonist to Type I Interferon▽

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Upon viral infection, the major defense mounted by the host immune system is activation of the interferon (IFN)-mediated antiviral pathway, which is mediated by IFN regulatory factors (IRFs). In order to complete their life cycle, viruses must modulate host IFN-mediated immune responses. Despite its association with significant human health problems, activities of Epstein-Barr virus (EBV), a human tumor-inducing herpesvirus, to evade host IFN-mediated innate immunity have not been well characterized. To search for EBV genes that block IFN signal transduction, we carried out a screening of EBV open reading frames for their abilities to block IFN-α/β-mediated luciferase expression upon Sendai virus infection. This screening demonstrates that EBV LF2 tegument protein specifically interacts with the central inhibitory association domain of IRF7, and this interaction leads to inhibition of the dimerization of IRF7, which suppresses IFN-α production and IFN-mediated immunity. This demonstrates a novel immune evasion mechanism of EBV LF2 in blocking cellular IRF7-mediated innate immunity.

The innate immune response is the host's front line of defense against microbial infection (15). Central to the host antiviral response is the production of type I interferon (IFN), which is delicately regulated by members of the IFN regulatory factor (IRF) family (5, 15, 21–23). This family has been implicated in antiviral defense, immune regulation, cell growth regulation, and apoptosis (3, 18, 33). The distinguishing characteristic of this family is the highly conserved, amino-terminal DNA-binding domain (DBD). Two closely related members of this family, IRF3 and IRF7, appear to be the main transducers of virus-mediated signaling in the induction of type I IFN (19, 22, 23, 27, 35). The transcription activity of IRF3 and IRF7 depends on the C-terminal phosphorylation, mediated by IKK-related kinases TBK1 and IKKε (12, 16, 36). Phosphorylation triggers series of alterations in IRF3 and IRF7, including conformation change, dimerization through a unique C-terminal domain known as the inhibitory associated domain (IAD), and nuclear translocation. These alterations result in the binding of DNA to IRF3 and IRF7 through their exposed DBD, which ultimately activates type I IFN transcription (28, 30, 39).

While IRF3 is a ubiquitous protein, IRF7 is IFN inducible and dominantly exists in lymphoid origin cells (1, 2, 4). Upon viral infection, IFN-β, whose expression is mainly regulated by IRF3, is thought to produce first due to its ubiquitous expression. IFN-β, upon binding to the IFN receptor, activates a signal cascade that eventually results in the transcriptional induction of hundreds of critical antiviral genes, including IFN-inducible protein kinase R, 2′,5′-oligoadenylate synthetases, TLR3, TLR7, and IRF7 (11, 35). The transcription of IFN-α, which is primarily regulated by IRF7, is highly activated as a result of the upregulation of IRF7 gene expression. Subsequently, secreted IFN-α induces another round of IFN receptor-mediated signal transduction as a positive feedback mechanism.

Most viruses have evolved strategies to defend themselves against host IFN responses (13, 15). These strategies include inhibiting IFN signaling by downregulating JAK-STAT signal molecule basal levels, suppressing particular molecular modifications, and preventing molecular translocation. For example, Ebola virus VP35 abolishes type I IFN production by inhibiting IRF3 activation (6, 7). Within Kaposi's sarcoma-associated herpesvirus (KSHV), a prototype gamma-2 herpesvirus, open reading frame 45 (ORF45) encodes a protein to block type I IFN production by inhibiting the phosphorylation and nuclear localization of IRF7 (40). In addition, KSHV vIRF3, called latency-associated nuclear antigen 2 (LANA2), was recently reported to significantly subvert type I IFN production by physically binding to IRF7 (24). Herpes simplex virus, a prototype alphaherpesvirus, encodes at least two modulators of IFN response, US11 and ICP34.5, which target a similar IFN response pathway, the double-stranded RNA-dependent protein kinase R pathway (8–10, 31).

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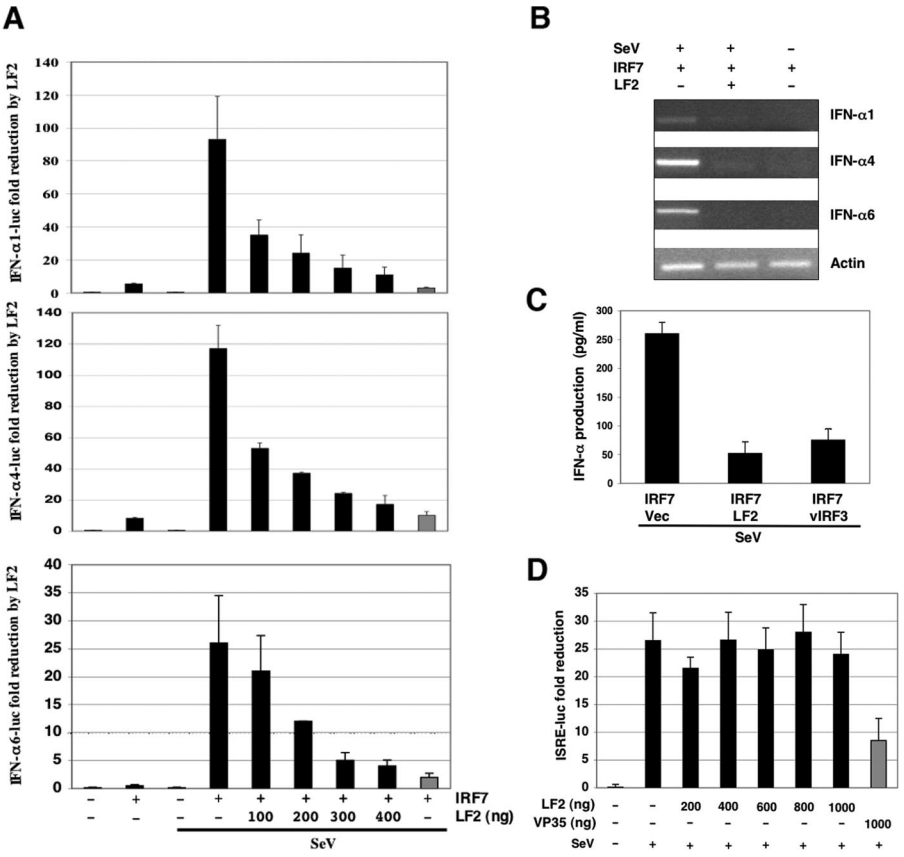

FIG. 1. LF2 inhibits type I IFN production. A total of 150 EBV entry clones constructed by Juergen Hass were subcloned into a destination vector called pCR3.1-flag/6xhis-dest to establish an EBV expression library by using Gateway LR Clonase (Invitrogen, San Diego, CA). Restriction enzyme digestion and DNA sequencing confirmed all of the EBV clones in pCR3.1-flag/6xhis-dest. Viral protein expression was determined by immunoblotting with anti-Flag antibody (Sigma, Saint Louis, MO). EBV LF2 sequence was then amplified from its entry clone and subcloned into the V5-tagged pCDNA5/FRT/To vector (Invitrogen) between the BamHI and XhoI sites. (A) LF2 inhibits the IRF7-induced activity of type I IFN promoters. 293T cells were transfected with a type I IFN (IFN-α1, IFN-α4, or IFN-α6) promoter-directed luciferase (luc) reporter, *Renilla* luciferase reporter, and IRF7 expression vectors along with increasing amounts of LF2 expression vector. At 24 h posttransfection, cells were infected with 50 HA units of SeV for 12 h. At 24 h postinfection, luciferase activity was measured by a dual-luciferase reporter assay kit from Promega Biotech (Madison, WI) and normalized to *Renilla* luciferase activity to standardize transfection efficiency. Results are the averages of four independent experiments. Four hundred nanograms of Ebola virus VP35 (the far-right bar in the panels) was used as a positive control in the experiment. (B) Suppression of SeV-induced IFN mRNAs by EBV LF2. A total of 1×10^5 293T cells were seeded into six-well plates before transfection and subsequently transfected using IRF7 with or without LF2, followed by stimulation with 100 HA units of SeV for 12 h. Real-time quantitative RT-PCR was performed to detect mRNA levels of type I IFN, as previously described (24). (C) Suppression of SeV-induced IFN-α production by EBV LF2. 293T cells were transfected with IRF7 expression vectors with or without LF2 expression vector. At 24 h posttransfection, cells were infected with 100 HA units of SeV for an additional 12 h. To detect IFN-α levels, cell culture media were collected for ELISA. The KSHV vIRF3 was included as a control. The results are the average of three independent experiments. (D) LF2 does not affect the IRF3-induced activation of ISRE promoter activity. 293T cells were transfected with an ISRE promoter-directed luciferase reporter and *Renilla* luciferase reporter along with increasing amounts of LF2. Cells were then treated with 100 HA units of SeV for 12 h. Luciferase activity was measured as described in Fig. 1A. Ebola virus VP35 was included as a control (shown by the last bar in the panel).

Epstein-Barr virus (EBV) is a ubiquitous DNA virus: 90% of the human population is infected with it (25). After infection, the virus will remain with the host for the rest of its life. EBV primary infection leads to infectious mononucleosis, while long-term exposure to EBV has no obvious symptoms in an immunocompetent host. In addition, EBV associates with a variety of tumors, including immunoblast lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, Burkitt's lymphoma, and gastric carcinoma in immunocompromised AIDS patients and organ transplant recipients under immunosuppressive treatment (25, 26). This indicates EBV is under the tight control of the host immune system. Two EBV proteins have been described which significantly suppress adaptive immune responses (20, 34). EBV BGLF5 helps the virus escape host T-cell recognition and elimination of the infected cell by shutting off the expression of major histocompatibility complex (MHC) class I and MHC class II genes (34). BNLF2a, an EBV lytic cycle early protein, blocks MHC class I presentation through inactivation of the TAP1/TAP2 peptide transporter to impair $CD8^+$ T-cell response (20). EBV BZLF1 was recently reported to negatively regulate IRF7, but the mechanism remains unclear (17). Despite its association with various human health problems, our knowledge of the EBV evasion strategy against type I IFN-mediated host innate immunity is lacking, especially compared to those of other herpesviruses.

To search for specific EBV genes that block IFN signal

transduction, we carried out a screening of EBV ORFs for their abilities to block IFN-α/β-mediated luciferase expression upon viral infection. To establish an EBV expression library, 150 EBV entry clones were subcloned into pCR3-Flag-His_6 destination vectors using the Gateway system. Each viral clone was examined by restriction enzyme digestion and DNA sequencing, while viral protein expression was determined by immunoblot analysis (data not shown). To identify EBV proteins that inhibit type I IFN production, 293T cells were transfected with IRF7, IFN-α6 promoter-directed luciferase reporter, and a transfectional control *Renilla* luciferase reporter along with individual EBV expression clones, and were subsequently infected with 50 hemagglutinating (HA) units of Sendai virus (SeV), a potent stimulus of type I IFN production. At 24 h postinfection, cells were harvested for reporter assays. Ebola virus VP35, which has been shown to block IFN signal transduction (6, 7), was included as a positive control. This screening discovered that EBV LF2 significantly inhibited SeV-induced activation of IFN-α6 promoter activity. To determine whether the inhibitory ability of LF2 was general or specific to IRF7, we tested its effects on the activation of IFN-α1, -4, and -6 promoters, which are regulated primarily by cellular IRF7 (11, 19, 22, 23, 27, 35). The results showed that, in a dosage-dependent manner, LF2 expression robustly suppressed IRF7-induced activation of IFN-α1, -4, and -6 promoter activities (Fig. 1A). To further address the inhibition of IFN promoter activity by LF2, we measured the mRNA levels of type I IFNs in the presence or absence of LF2 expression. At 24 h posttransfection using IRF7, with and without EBV LF2, 293T cells were infected with SeV for 16 h and then harvested for the isolation of total RNAs. Reverse transcription-PCR was performed with equal amounts of total RNA to determine the type I IFN mRNA levels. Upon SeV infection, a marked increase of IFN-α1, IFN-α4, and IFN-α6 mRNAs was detected in cells transfected with IRF7 vector, whereas an increase was minimal or undetectable in cells transfected with both IRF7 and LF2 (Fig. 1B). Furthermore, enzyme-linked immunosorbent assay showed that similar to KSHV vIRF3, EBV LF2 effectively suppressed IFN-α secretion induced by SeV infection (Fig. 1C). These results indicate that EBV LF2 efficiently blocks the IRF7-mediated activation of type I IFN expression.

While both IRF7 and IRF3 are critical transcription regulators of type I IFN gene expression, IRF7 primarily activates IFN-α1, -4, -6, and -14 promoter activity and IRF3 induces ISRE (IFN-stimulated responsive element) promoter activity (11, 19, 22, 23, 35). We investigated whether LF2 expression abrogated the IRF3-induced activation of ISRE promoter activity. At 24 h posttransfection with ISRE promoter-directed luciferase reporter with increasing amounts of LF2, 293T cells were infected with SeV for 12 h and then harvested for a luciferase assay. The results showed that, unlike its robust abrogation of IRF7 transcriptional activity, LF2 demonstrated no effect on IRF3-induced activation of ISRE promoter activity (Fig. 1D). In contrast, Ebola virus VP35 considerably suppressed virus-induced ISRE promoter activity under the same conditions (Fig. 1D). These results suggest that EBV LF2 specifically inhibits the activity of IRF7 but not IRF3.

Given the inhibitory effect of EBV LF2 on IRF7-induced transcription, we tested the potential interaction of LF2 with IRF7. At 48 h posttransfection with IRF7 and Flag-tagged LF2 expression vectors or Flag-tagged IRF7 and V5-tagged LF2 expression vectors, 293T cells were used for immunoprecipitation (IP) with anti-Flag antibody, followed by immunoblotting with anti-IRF7 or anti-V5 antibody, respectively. Co-IPs showed efficient interaction between LF2 and IRF7 (Fig. 2A and B). Despite a high degree of similarity with IRF7, IRF3 was not able to bind to LF2 under the same conditions (Fig. 2C). Taken together, these data demonstrate that LF2 specifically interacts with IRF7, but not IRF3, and this interaction most likely accounts for the ability of LF2 to block IRF7-mediated activation of type I IFN expression.

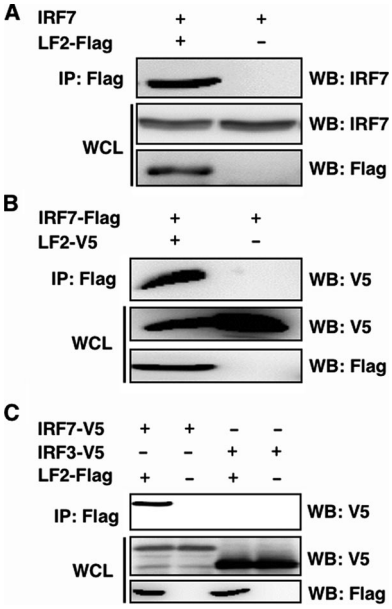

FIG. 2. (A and B) LF2 interaction with IRF7. 293T cells were transfected with IRF7 and/or Flag-LF2 (A) or Flag-IRF7 and/or V5-LF2. At 48 h posttransfection, cells were harvested for IP with anti-Flag antibody, followed by Western immunoblotting (WB) with anti-IRF7 antibody (A) or anti-V5 antibody (B). (C) LF2 does not interact with IRF3. 293T cells were transfected with Flag-LF2 and either V5-IRF7 or V5-IRF3 for Co-IP as described above. WCL, whole-cell lysates.

Upon viral infection, IRF7 undergoes an infection-induced serine phosphorylation within its carboxyl-terminal regulatory domain. This modification subsequently stimulates protein dimerization, nuclear retention, and interaction with transcriptional coactivators, resulting in the activation of a robust response comprised of a broad spectrum of IFN isotypes (28, 30). As previously shown (12, 16, 36), expression of TBK1 and IKKε kinases, which phosphorylate the carboxyl-terminal regulatory domain of IRF7, led to the activation of IRF7 transcriptional activity, resulting in a dramatic increase of IFN-α4 promoter activity (Fig. 3A). However, LF2 coexpression effectively suppressed TBK1 or IKKε kinase-mediated activation of IRF7 transcriptional activity (Fig. 3A). Under these conditions, IKKε or TBK coexpression led to an almost complete shift of IRF7 to its slow-migrating, phosphorylated form during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3B). We found that LF2 had no effect on TBK- or IKKε-mediated migration retardation of IRF7 (Fig. 3B). A mutation of the C-terminal 477 and 479 serine residues of IRF7 to

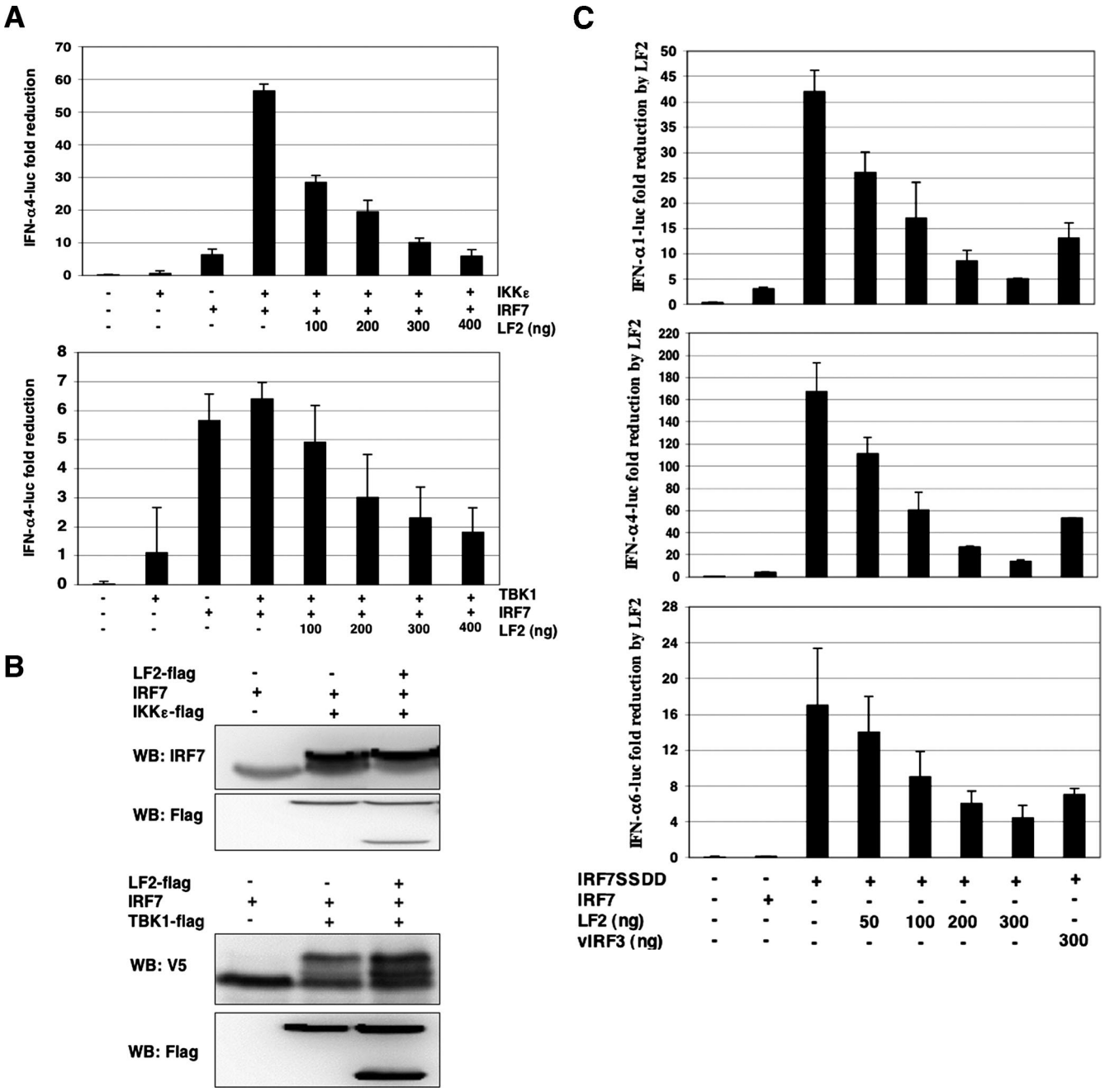

FIG. 3. LF2 expression does not affect IRF7 phosphorylation. (A) LF2 downregulates the IKKε- or TBK1-mediated activation of IRF7 transcriptional activity. 293T cells were transfected with an IFN-α4 promoter-directed luciferase (luc) reporter, *Renilla* luciferase reporter, IRF7 vector, and IKKε (the upper panel) or TBK1 (the lower panel) vector along with increasing amounts of LF2 vector. Luciferase activity was measured as described in Fig. 1A. The IRF7 expression level was monitored by immunoblot analyses (one example shown below). (B) LF2 does not inhibit the IRF7 phosphorylation induced by IKKε or TBK1. 293T cells were transfected with Flag-IKKε or Flag-TBK1 and IRF7 with or without Flag-LF2. Western immunoblot (WB) analysis was then performed with the appropriate antibodies. (C) LF2 impairs the activity of type I IFN promoter activities induced by constitutively active IRF7 S477D S479D mutant. 293T cells were transfected with an IFN promoter (IFN-α1, IFN-α4, or IFN-α6)-directed luciferase reporter, *Renilla* luciferase reporter, and IRF7 S477D S479D along with increasing amounts of LF2. Luciferase activity was measured as described in Fig. 1. KSHV vIRF3 was included as a control.

negatively charged glutamic acid, called IRF7 S477D S479D, has been shown to mimic TBK- or IKKε-mediated phosphorylation, resulting in the constitutive activation of IRF7 transcriptional activity (28, 30, 39). LF2 suppressed, in a dosage-dependent manner, the transcriptional activity of IRF7 S477D S479D as effectively as that of wild-type IRF7 (Fig. 3C). Furthermore, LF2 did not affect IR7 nuclear translocation because LF2 is localized in the nucleus as well (Fig. 4). Finally, despite the fact that IRF7 undergoes a proteosomal degradation pathway (38), LF2 expression did not affect IRF7 protein levels at

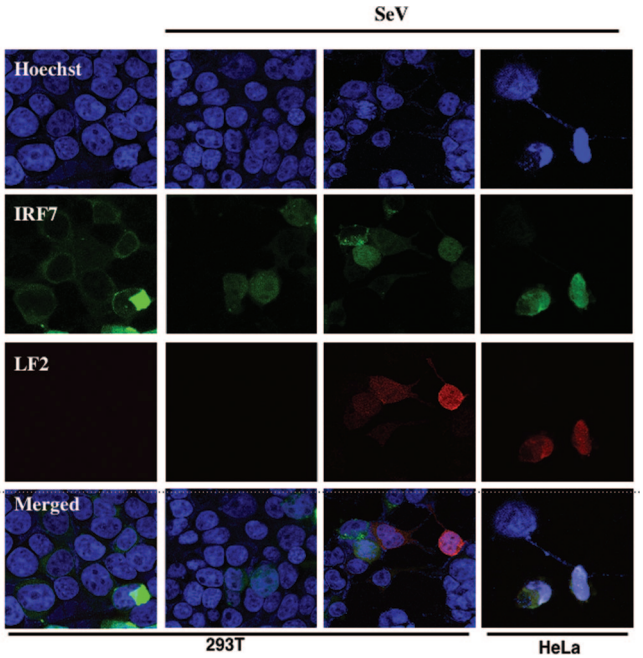

FIG. 4. LF2 expression does not affect IRF7 nuclear translocation. 293T cells or HeLa cells were transfected with either Flag-tagged IRF7 alone or together with V5-tagged LF2. Cells then were infected with SeV at 12 h posttransfection. Immunostaining was performed on fixed cells at 6 h postinfection, and confocal microscopy was used to observe the fluorescence.

a detectable level (Fig. 5). These results collectively indicate that LF2 interaction has no effect on IRF7 phosphorylation and protein stability.

IRF7 consists of an N-terminal tryptophan repeat containing a DBD, a transactivation domain, an IAD, and a C-terminal regulatory domain (RD) (28, 30, 39). The virus-induced phosphorylation of IRF7 appears to relieve an intramolecular association between two autoinhibitory domains and unmask the N-terminal DBD and C-terminal IAD, resulting in the formation of homodimers through the IAD. IRF7 dimers subsequently translocate from the cytoplasm to the nucleus and stimulate DNA binding and transcriptional activities (28, 30, 39). To test whether LF2 interaction blocked IRF7 dimerization, glutathione *S*-transferase (GST)-IRF7 fusion and Flag-IRF7 proteins were coexpressed in 293T cells along with increasing amounts of V5-LF2. A GST pull-down assay showed that GST-IRF7 and Flag-IRF7 homodimerization was apparently reduced upon LF2 expression, while GST-IRF7 and V5-LF2 interaction increased under the same conditions (Fig. 6A). Cells were transfected with a V5-LF2 expression vector and GST-IRF7 fusions containing the DBD, activation domain (AD), IAD, RD, or the full-length gene, followed by GST pull-down. The pull-down assay showed that V5-tagged LF2 efficiently interacted with full length GST-IRF7 and GST-IAD but not with GST, GST-DBD, GST-AD, and GST-RD (Fig. 6B). These results indicate that LF2 effectively inhibits IRF7 dimerization by interacting with the IAD of IRF7, which may account for the impairment of IRF7-induced transcription.

IRF7 is a master regulator of innate immunity and also plays an important role in the transition from innate immunity to acquired immunity (22). Due to its essential role in host im-

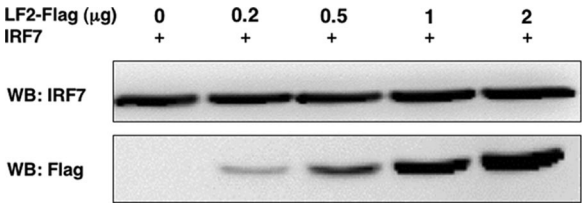

FIG. 5. LF2 expression does not affect IRF7 protein level. 293T cells were transfected with IRF7 vector and increasing amounts of Flag-LF2 vector. Cells were collected for Western immunoblot (WB) analysis to detect the expression levels of IRF7 and LF2 using the appropriate antibodies.

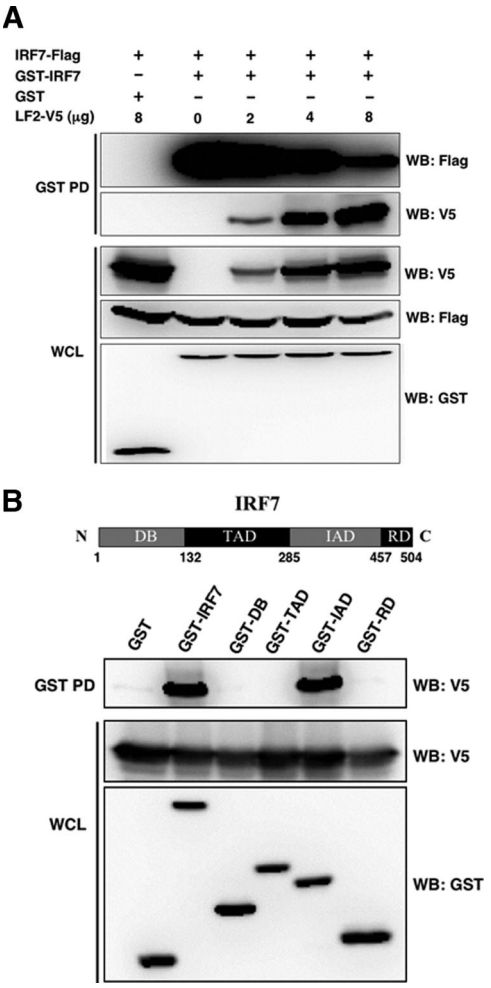

FIG. 6. LF2 blocks the IRF7 dimerization by binding to the IAD of IRF7. (A) LF2 blocks the IRF7 dimerization. 293T cells were transfected with GST-IRF7 and Flag-IRF7 along with increasing amounts of V5-LF2. GST pull-down (PD) was performed at 36 h posttransfection, followed by Western immunoblotting (WB) with anti-Flag or anti-V5 antibody. Whole-cell lysates (WCL) were included in the immunoblot assay. (B) LF2 binds to the IAD of IRF7. IRF7 domains have been shown in the schematic diagram. Each domain of IRF7 was in-frame fused with mammalian GST. At 36 h postransfection with GST-IRF7 fusion vector and V5-LF2 vector, 293T cells were harvested for GST pull-down, followed by immunoblotting with anti-V5 antibody.

munity, numerous viruses have developed various strategies to downregulate IRF7 activation. ICP0, encoded by herpes simplex virus and bovine herpesvirus 1, inhibits phosphorylation by TBK1 and IKKε (29). In response to viral infection, a KSHV immediate-early lytic protein encoded by ORF45 that is a virion tegument protein binds to IRF7 and blocks its phosphorylation and accumulation in the nucleus, resulting in a blockage of IFN-α and IFN-β transcription (40). In addition, the KSHV RTA immediate-early lytic nuclear transcription factor acts as an ubiquitin E3 ligase to promote the ubiquitination and degradation of IRF7 protein in a proteasome-dependent manner (38). In addition, KSHV vIRF3 has been recently shown to bind IRF7, which suppresses the DNA binding ability of IRF7 (24). Our present study adds EBV LF2 to the expanding family of viral proteins that inhibit cellular IRF7 transcriptional activity through protein-protein interactions.

EBV-encoded dUTP nucleotidohydrolase (dUTPase) has recently been reported to induce immune dysregulation in vivo in mice (14). EBV LF1 (ORF10) and LF2 (ORF11) both contain a dUTPase-like domain, suggesting the potential role of a dUTPase-like domain for anti-IFN activity. However, our preliminary study indicated that a dUTPase-like domain was not required for the inhibition of type I IFN production by LF2 (unpublished data). In addition, EBV LF1 and LF2 do not appear to be essential for viral replication in cell culture, since the genomic region containing these sequences is deleted from the EBV B95-8 strain frequently used to study viral replication and immortalization. Furthermore, we also found that the LF2 gene of rhesus lymphocryptovirus, the virus most closely related to EBV (32, 37), efficiently downregulates IRF7-induced transcription of type I IFN, suggesting genetic and functional conservation of LF2 in these two viruses (unpublished data). Construction of LF2-knockout rhesus lymphocryptovirus would be useful in elucidating the functional significance of LF2 as a type I IFN antagonist, therefore providing a better understanding of whether and how LF2 contributes to successful EBV infection and pathogenesis in vivo.

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